

# Pyrimido[1,2-*a*]-purin-10(3*H*)-one, M<sub>1</sub>G, is less prone to artifact than base oxidation

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## ABSTRACT

**Pyrimido[1,2-*a*]-purin-10(3*H*)-one (M<sub>1</sub>G) is a secondary DNA damage product arising from primary reactive oxygen species (ROS) damage to membrane lipids or deoxyribose. The present study investigated conditions that might lead to artifactual formation or loss of M<sub>1</sub>G during DNA isolation. The addition of antioxidants, DNA isolation at low temperature or non-phenol extraction methods had no statistically significant effect on the number of M<sub>1</sub>G adducts measured in either control or positive control tissue samples. The number of M<sub>1</sub>G adducts in nuclear DNA isolated from brain, liver, kidney, pancreas, lung and heart of control male rats were 0.8, 1.1, 1.1, 1.1, 1.8 and 4.2 M<sub>1</sub>G/10<sup>8</sup> nt, respectively. In rat liver tissue, the mitochondrial DNA contained a 2-fold greater number of M<sub>1</sub>G adducts compared with nuclear DNA. Overall, the results from this study demonstrated that measuring M<sub>1</sub>G is a reliable way to assess oxidative DNA damage because the number of M<sub>1</sub>G adducts is significantly affected by the amount of ROS production, but not by DNA isolation procedures. In addition, this study confirmed that the background number of M<sub>1</sub>G adducts reported in genomic DNA could have been overestimated by one to three orders of magnitude in previous reports.**

## INTRODUCTION

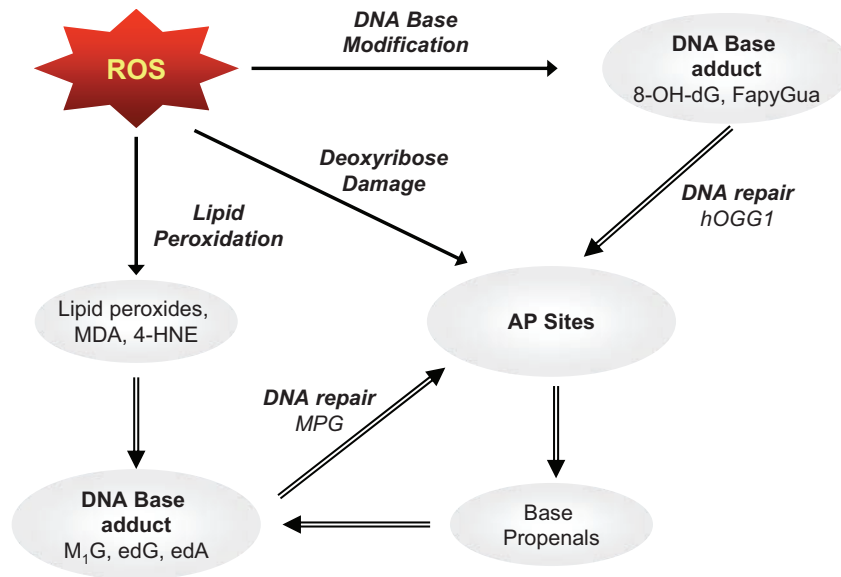
Reactive oxygen species (ROS), primarily superoxide anion radical (O<sub>2</sub><sup>-</sup>), are produced naturally from cellular sources such as mitochondria (1,2). In addition, humans, wildlife and laboratory animals are exposed daily to a complex mixture of chemicals, primarily via trace amounts present in food, drinking water or air, that can contribute to increased cellular ROS through a variety of mechanisms (3). Various oxidative DNA

lesions (Scheme 1) have been characterized and investigated for their role in the pathogenesis of adverse health effects and disease. One of the most studied biomarkers for oxidative DNA damage is 7,8-dihydro-8-OH-2'-deoxyguanosine (8-OH-dG). It is produced in relatively high amounts in cells and sensitive methods (i.e. LC-MS/MS) are available to measure it. However, the measurement of a single lesion such as 8-OH-dG as a biomarker might be misleading because it may not reflect the overall spectrum of DNA damage and its potential for mutations. In addition, the accumulation of oxidative DNA lesions could be affected by DNA repair, which is mediated by different pathways depending on the type of DNA lesion. Therefore, it is desirable to have multiple biomarkers to monitor oxidative DNA damage for investigating the role of ROS in the pathogenesis of diseases.

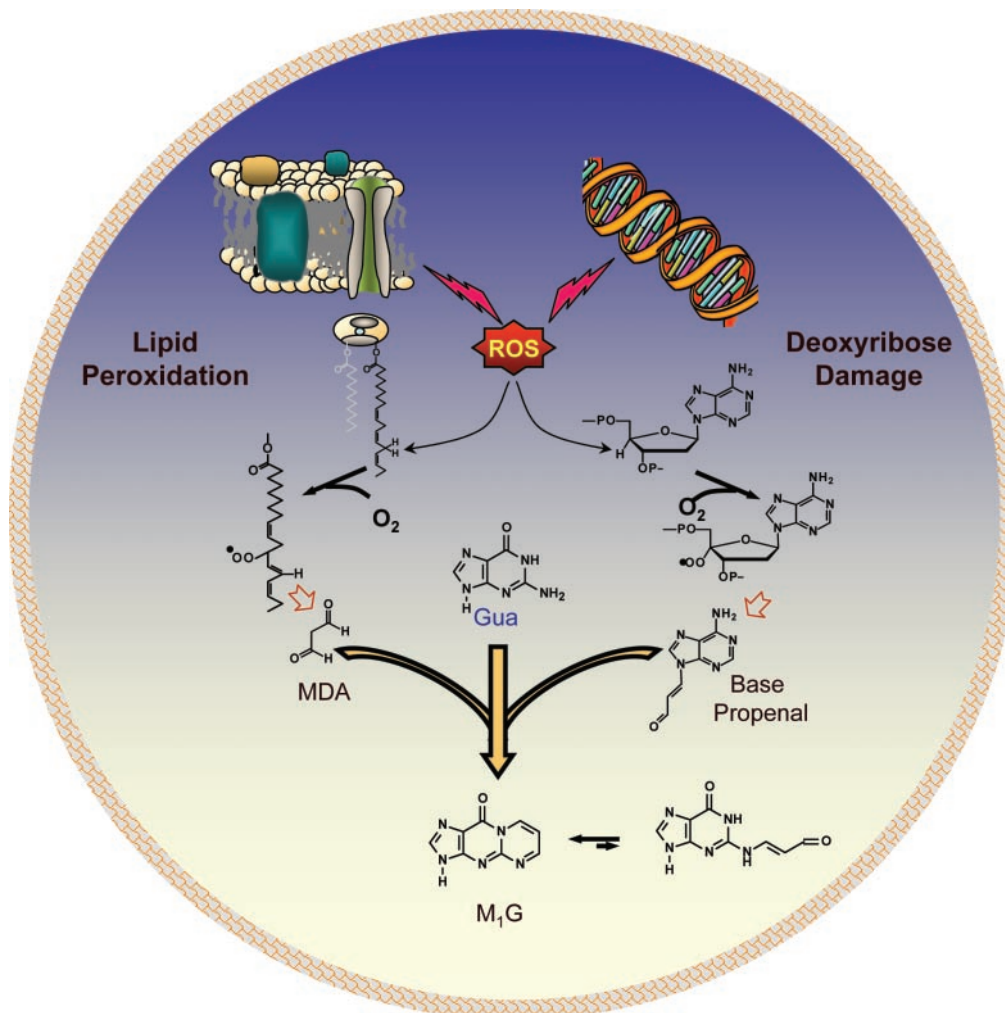
In addition to primary ROS lesions, a growing body of evidence supports that secondary DNA lesions produced from byproducts of primary DNA damage or lipid membrane oxidation play an important role in mutation (4,5) (Scheme 1). Pyrimido[1,2-*a*]-purin-10(3*H*)-one (M<sub>1</sub>G) is one example of a secondary DNA lesion formed by primary ROS insults to lipid membranes or the DNA backbone (6–8) (Scheme 2). Malondialdehyde (MDA) from lipid peroxidation has been considered to be a key intermediate in M<sub>1</sub>G formation (8). Dedon *et al.* (6) proposed an alternative pathway leading to M<sub>1</sub>G formation via site-specific free radical attack on the DNA backbone.

Owing to its relatively high mutagenicity (9) and endogenous presence (10–14), considerable effort has been expended to establish a reliable method to measure M<sub>1</sub>G adducts in genomic DNA. However, wide variation in the number of endogenous M<sub>1</sub>G adducts has been reported for animal tissues. For example, different research groups (12,15–17) have reported between 4 and 300 M<sub>1</sub>G/10<sup>7</sup> nt from rat liver DNA. Such a wide variation in endogenous M<sub>1</sub>G adducts poses great difficulty for using this biomarker in epidemiological studies. There is a critical need to establish normal and abnormal ranges of M<sub>1</sub>G adducts as indicators of oxidative stress induced by diet, environmental or occupational exposures. A potential cause for such large discrepancies in the number

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**Scheme 1.** Primary ( $\rightarrow$ ) and secondary ( $\Rightarrow$ ) DNA damage induced by ROS. FapyGua, 2,6-diamino-4-hydroxy-5-formamido-pyrimidine; hOGG1, 8-oxoguanine DNA glycosylase; 4-HNE, 4-hydroxy-2-nonenal; MPG, N-methylpurine-DNA-glycosylase; edG, ethenodeoxyguanosine; edA, 1,N<sup>6</sup>-ethenodeoxyadenosine.



**Scheme 2.** Formation of M<sub>1</sub>G from ROS and equilibrium of M<sub>1</sub>G.

of endogenous oxidative DNA lesions is artifacts that arise during DNA isolation and sample analysis. To investigate the role of M<sub>1</sub>G in cancer development, our research group has established a novel analytical approach to measure M<sub>1</sub>G using selective labeling with aldehyde reactive probe (18). This approach significantly reduced the artifacts during M<sub>1</sub>G analysis by producing a stable conjugate, preventing artifactual formation or loss of adducts, enhancing sample enrichment, and finally measurement by LC-MS/MS. However, the production of artifacts during DNA isolation has not been investigated thoroughly for M<sub>1</sub>G.

Years of research on primary oxidative DNA damage have shown that great care must be taken to avoid artifactual oxidative DNA damage during DNA isolation (19,20). Since M<sub>1</sub>G is a product of ROS DNA damage, it is possible that artifactual formation of M<sub>1</sub>G during DNA isolation could mask actual amounts of this lesion produced from either endogenous metabolism or chemical exposures. Furthermore, ring-opened M<sub>1</sub>G, which is the dominant form in genomic DNA, is reactive toward amine compounds including Tris, a common buffering reagent used in DNA isolation (21,22). Conjugate formation between M<sub>1</sub>G and amines could cause false negative results by decreasing the number of adducts recovered after DNA isolation. Therefore it is critical to establish a dependable DNA extraction protocol that minimizes errors in the measurement of M<sub>1</sub>G. These considerations led us to investigating the influence on M<sub>1</sub>G adduct quantification of artifacts during DNA isolation from tissue. In this study, artifactual formation of M<sub>1</sub>G during DNA isolation was examined by comparing the following effects on the number of M<sub>1</sub>G adducts: presence of added antioxidants; temperature during the work-up procedure; and different DNA isolation methods (Table 1). The loss of M<sub>1</sub>G adducts during DNA isolation was tested by the addition of positive control DNA which had 25 times larger amounts of M<sub>1</sub>G adducts than control calf thymus DNA. In addition, the quality of DNA was examined by monitoring DNA yield and the A<sub>260</sub>/A<sub>280</sub> ratio. Finally, DNA was isolated from the organs of control male rats with the newly established DNA isolation protocol for subsequent M<sub>1</sub>G analyses. The numbers of M<sub>1</sub>G adducts were then compared between the organs and with data from previous reports.

## MATERIALS AND METHODS

### Materials and instrumentation

Absolute alcohol was purchased from Aaper ethanol (Shelbyville, KY). Other solvents were high-performance liquid chromatography (HPLC) grade and were purchased

from Fisher Scientific (Raleigh, NC). Snap frozen rat tissues were purchased from Taconics (Germantown, NY). Proteinase K, lysis buffer and 70% phenol solutions were purchased from Applied Biosystems (Foster City, CA). Unless stated otherwise, all other chemicals and enzymes were purchased from Sigma-Aldrich Chemical Company (St Louis, MO) and were used without further purification. To prepare homogeneous tissue samples, frozen liver tissues from five control male rats were pooled together and ground into powder using a pre-chilled (−80°C) mortar and pestle. The tissue powder was distributed into small tubes and kept in a −80°C freezer until use.

Quantification of conjugates of M<sub>1</sub>G with pentafluorobenzyl hydroxylamine (PFBHA) and its internal standard were performed using a Finnigan Quantum (Thermo, Woburn, MA) triple-quadrupole mass spectrometer connected to the Finnigan Surveyor Micro-LC (Thermo, Woburn, MA).

### Effect of antioxidants on M<sub>1</sub>G formation during DNA isolation

*Method A.* DNA was extracted by a procedure described by Gupta (23) with minor modifications. Briefly, 1 g frozen tissue powder was reconstituted in 7 ml homogenization buffer consisting of phosphate-buffered saline (PBS; pH 7.4) with or without an antioxidant. The tissue samples were homogenized with a Tehran homogenizer (Wheaton Instruments, Millville, NJ). After centrifugation at 1000 g for 15 min, the precipitate was washed with 7 ml homogenization buffer. The nuclear fraction was collected by centrifugation and was reconstituted in 6 ml lysis buffer (Applied Biosystems). An RNase mixture (60 μl) consisting of RNase A (80 KeU/ml) and RNase T1 (0.3 U/ml) was added to the tissue lysate, followed by incubation for 1 h at 37°C. Proteinase K (400 U/ml, 60 μl) was added to the samples and they were further incubated for an hour at 50°C. Hydrolyzed protein was then extracted twice with 6 ml of 70% phenol solution (Applied Biosystems) and once with 6 ml chloroform:isoamyl alcohol (49:1, Sevag solution). DNA was precipitated from the aqueous layer by sequential addition of 300 μl 3 M NaCl and 12 ml cold ethanol. After the DNA was collected by centrifugation, the DNA pellet was rinsed with 6 ml of 70% ethanol and air dried on ice. The DNA pellet was resuspended in 500 μl HPLC grade water. DNA samples (25 μl) were mixed with 975 μl of 20 mM Tris-EDTA buffer (pH 8.0) to measure DNA concentration and purity by UV. The DNA solution was stored at −80°C until M<sub>1</sub>G analysis. To investigate the artifactual formation of M<sub>1</sub>G by oxidation of deoxyribose or lipids during DNA isolation, either butylated hydroxytoluene (BHT), 2,2,6,6-tetramethylpiperidine 1-oxyl

**Table 1.** Different methods used for DNA isolation from rat liver tissue

Method	Description	Antioxidant	Tissue fraction	Buffering reagent	Protein removal
A	DNA isolation at room temperature	BHT, Tempo, Desferal or Hydroxylamine	Nuclear fraction	Tris	Phenol extraction
B	DNA isolation at low temperature	Tempo, Desferal, Hydroxylamine or PFBHA	Nuclear fraction	Tris	Phenol extraction
C	DNA isolation from tissue homogenate	None	Tissue homogenate	HEPES	Phenol extraction
D	DNA isolation with Tris	Tempo	Nuclear fraction	Tris	Phenol extraction
E	DNA isolation with HEPES	Tempo	Nuclear fraction	HEPES	Phenol extraction
F	DNA isolation with protein precipitation	Tempo	Nuclear fraction	HEPES	Protein precipitation with NaCl
G	DNA isolation with NaI	Tempo	Nuclear fraction	HEPES	Chaotropic reagent (NaI)

(TEMPO), desferal or hydroxylamine was added to the buffer solutions. Homogenization buffer, lysis buffer, 70% phenol and Sevag solutions were supplemented with the additives in the following final concentrations: BHT, 0.01, 0.01, 0.5 and 0.5%; TEMPO, 10, 10, 10 and 10 mM; desferal, 1, 1, 1 and 1 mM; and hydroxylamine, 10, 2, 2 and 2 mM, respectively. To investigate the loss of M<sub>1</sub>G during DNA isolation, a positive control sample was prepared by the addition of MDA treated CTD (18) into the nuclear fraction. The concentration of M<sub>1</sub>G in the positive control DNA was calculated to be 80 fmol M<sub>1</sub>G/100 mg tissue which corresponded to 25 M<sub>1</sub>G/10<sup>8</sup> nt based on a DNA yield of 100 µg/100 mg tissue. Tissue powder (100 mg) was homogenized in 700 µl homogenization buffer and the nuclear fraction was collected and washed as described previously. The nuclear pellet was reconstituted in the lysis buffer followed by the addition of 40 µl MDA-CTD solution (2 fmol M<sub>1</sub>G/µl, 0.4 ng DNA/µl). DNA was isolated and stored as described previously. In addition to the antioxidants used for the control tissue, PFBHA was tested and compared for its effect on artifactual formation or loss during DNA isolation. The final concentration for PFBHA in the reagents for DNA isolation was 2 mM.

#### DNA isolation at low temperature for M<sub>1</sub>G adducts measurement

*Method B.* To investigate the effect of temperature during DNA isolation on the number of M<sub>1</sub>G adducts, DNA was extracted from control liver tissue at low temperature (4°C) with a combination of antioxidants, a chelator or alkoxyamines. DNA was extracted by a procedure described by Nakamura and Swenberg (24) with minor modifications. Briefly, the nuclear fraction was prepared from frozen tissue and reconstituted in lysis buffer as described above with pre-chilled solutions and instruments. Proteinase K (400 U/ml, 60 µl) was added to the sample and incubated overnight at 4°C. Hydrolyzed proteins were extracted with phenol extractions and nucleic acids were precipitated by the addition of cold ethanol as detailed previously. The nucleic acid pellet was reconstituted in 2 ml RNA digestion buffer consisting of RNase A (0.8 KeU/ml), RNase T1 (3 mU/ml) and one of the antioxidants in 10 mM HEPES buffer (pH 7.8). The concentration of TEMPO, desferal, hydroxylamine or PFBHA was 1, 0.1, 0.1 or 0.1 mM, respectively. After 1 h incubation at 37°C, DNA was precipitated by the sequential addition of 100 µl of 3 M NaCl and 4 ml cold ethanol. The DNA was collected by centrifugation and rinsed with 70% ethanol. The DNA pellet was reconstituted in 500 µl HPLC grade water.

#### Comparison of DNA isolation methods for M<sub>1</sub>G adducts analysis

To investigate M<sub>1</sub>G formation or loss by different types of DNA isolation procedures, DNA was isolated from both control tissue and positive control samples with one of the methods detailed below.

*Method C.* To investigate the effect of the components within the cell cytosol on M<sub>1</sub>G adduct numbers, DNA was isolated from tissue homogenates without nuclear fraction isolation. After the homogenization of tissue in lysis buffer (10 mM HEPES, 1 mM EDTA, 1% SDS and 10 mM TEMPO), RNases and Proteinase K (4 U/ml to final concentration)

were sequentially added to the tissue homogenate. DNA was isolated as described in Method A.

*Method D.* To investigate the effect of Tris present in DNA isolation reagents on M<sub>1</sub>G, DNA was isolated in the presence of TEMPO according to the protocol outlined in Method B and was then analyzed for M<sub>1</sub>G adducts.

Frozen tissue powder (3 g) was reconstituted in 21 ml pre-chilled (4°C) homogenization buffer consisting of PBS and 10 mM TEMPO. The nuclear fraction was prepared from tissue homogenization followed by centrifugation. The isolated nuclear fraction was washed with homogenization buffer and reconstituted in 18 ml lysis buffer containing Proteinase K (4 U/ml), 10 mM HEPES, 1 mM EDTA, 1% SDS and 10 mM TEMPO in water. The samples were incubated overnight in a cold room (4°C) with mild shaking. The lysate was distributed to three tubes for DNA isolation via methods E, F and G.

*Method E.* Hydrolyzed protein was removed by extractions with HEPES-buffered phenol (pH 8.0) and Sevag solution as described in Method B. To prepare the HEPES-buffered phenol, ACS grade phenol was melted at 60°C. An equal volume of 0.1 M HEPES buffer (pH 8.0) was added to the phenol followed by a vigorous mixing for 30 min. The aqueous layer was separated from the phenol mixture after standing for 10 min. After the aqueous layer was replaced with fresh HEPES buffer, the phenol solution was extracted twice with 0.1 M HEPES buffer. The phenol solution was mixed with one-fifth volume of 0.1 M HEPES buffer containing 10 mM TEMPO and was used on the same day as DNA isolation. After the extraction of hydrolyzed proteins with the Sevag solution, nucleic acids were precipitated by sequential additions of NaCl and cold ethanol. The precipitated nucleic acids were collected by centrifugation and washed with 12 ml of 70% ethanol. The remaining ethanol was removed by air on ice for 15 min and the DNA pellet was reconstituted in 2 ml RNase buffer containing RNase A (0.3 KeU/ml), RNase T1 (3 mU/ml) and 10 mM TEMPO in 10 mM HEPES buffer. After an hour of incubation at 37°C, DNA was precipitated by the sequential addition of 100 µl of 3 M NaCl and 4 ml cold ethanol. Afterwards, DNA was collected by centrifugation and rinsed with 70% ethanol. The DNA pellet was resuspended in 500 µl HPLC grade water. DNA concentration was measured and the DNA was stored at -80°C until the M<sub>1</sub>G adducts assay.

*Method F.* In this method, the hydrolyzed proteins were precipitated from the sample by salting out as described previously (25,26) with minor modifications. A 2 ml aliquot of 5 M NaCl was added to the tissue lysate followed by vigorous shaking for 1 min. After incubation on ice for 10 min, the hydrolyzed proteins were removed by centrifugation at 4000 g for 20 min. The supernatant was added to 6 ml isopropanol in a new tube and gently mixed by inversion for 20 min at 4°C. The nucleic acid threads were collected by centrifugation and washed with 12 ml of 70% ethanol. The remaining ethanol was removed by air drying on ice for 15 min and the nucleic acids pellet was reconstituted in 2 ml RNase buffer for subsequent RNA removal followed by DNA precipitation as described in Method E.

*Method G.* DNA was fractionated with isopropanol in the presence of NaI and SDS as demonstrated in previous studies

(27,28). Tissue lysate (6 ml) was mixed with 12 ml of 6.75 M NaI solution containing 2 mM EDTA, 10 mM TEMPO and 10 mM HEPES (pH 8.0). The sample was mixed by manual inversion followed by the addition of 18 ml isopropanol. The sample was gently mixed in a cold room by an end-over-end mixer for 30 min. The precipitated nucleic acids were collected and washed with 18 ml of 70% ethanol. The following RNA digestion and DNA collection procedures were the same as described previously.

#### DNA isolation from rat tissues

Liver, kidney, pancreas, lung, heart and brain harvested from five control male rats in the age range of 7–8 months were used to isolate DNA. Nuclear and mitochondrial fractions were separated from liver tissue by using the protocol described by Anson *et al.* (29) with minor modifications. Liver tissues were thawed in seven times volume (v/w) of ice-cold MSHE buffer [0.21 M mannitol, 0.07 M sucrose, 10 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA and 10 mM TEMPO]. The tissue samples were homogenized with a Tehran homogenizer followed by centrifugation at 50 *g* for 5 min. The supernatant was decanted to the new tube followed by centrifugation at 1000 *g* for 10 min. The precipitated nuclear fraction was washed twice with MSHE buffer and processed for nuclear DNA isolation using Method F. The supernatant was subjected to centrifugation at 9000 *g* for 20 min. The precipitated mitochondrial fraction was washed twice by reconstitution in MSHE buffer followed by centrifugation. The isolated mitochondrial fraction was subjected to DNA isolation using the procedures for DNA isolation from nuclear fraction. The DNA pellet isolated from the mitochondrial fraction was reconstituted in 200  $\mu$ l water. Frozen lung or heart tissues were thawed in 10 times volume (v/w) of ice-cold homogenization buffer consisting of 10 mM TEMPO and PBS. The tissue samples were minced with scissors and a tissue tearer (Biospec Products Inc., Bartlesville, OK) followed by homogenization. The procedures for nuclear DNA isolation were the same as those for DNA isolation from liver nuclear DNA. Kidney, pancreas or brain tissue was thawed in seven times volume (v/w) of ice-cold homogenization buffer followed by a homogenization for nuclear fraction isolation. The nuclear fraction was subjected to the protocol used for DNA isolation from liver nuclear fraction.

#### Analysis of M<sub>1</sub>G adducts in DNA

The number of M<sub>1</sub>G adducts in sample DNA was determined by the specific derivatization of DNA with PFBHA and LC-MS/MS analysis for the PFBHA-M<sub>1</sub>G conjugate similar to the protocol described elsewhere (18). Briefly, 100  $\mu$ g of sample DNA was mixed with internal standard DNA (100 fmol <sup>15</sup>N<sub>5</sub>-M<sub>1</sub>G) in 900  $\mu$ l of 2 mM PFBHA solution. After 30 min at 70°C, 100  $\mu$ l of 0.1 M HCl was added to the sample mix. After another 30 min at 70°C, the sample was cooled to room temperature. A 10  $\mu$ l aliquot was mixed with 90  $\mu$ l water and used for purine analysis to measure DNA concentration as described in a previous report (18). The remaining sample was placed on an SPE column (HLB-60, Waters, Milford, MA), that had been preactivated and equilibrated with methanol and water, respectively. The SPE column was washed once with 2 ml water and twice

with 3 ml of 65% methanol. PFBHA-M<sub>1</sub>G and the internal standard were eluted with 3 ml of 90% methanol in water. The solvent was removed by vacuum evaporation. The samples were reconstituted in 40  $\mu$ l of 10% methanol in water and stored at –80°C until analysis. The samples were separated on an Atlantis d-C18 (3  $\mu$ m, 2 mm  $\times$  150 mm, Waters) HPLC column by a gradual increase of acetonitrile. Two specific ion transitions (i.e. *m/z* 401  $\rightarrow$  *m/z* 188 and *m/z* 406  $\rightarrow$  *m/z* 193) were used to detect and quantify PFBHA-M<sub>1</sub>G and PFBHA-<sup>15</sup>N<sub>5</sub>-M<sub>1</sub>G, respectively.

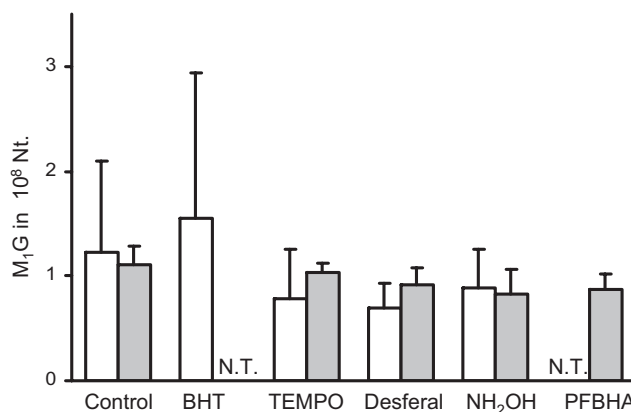
#### Data analysis

The presence of significant differences between mean values was determined by an analysis of variance (ANOVA). The level of statistical significance in all cases was *P* < 0.05.

## RESULTS

#### Effect of antioxidants or work-up temperature on the number of M<sub>1</sub>G adducts during DNA isolation

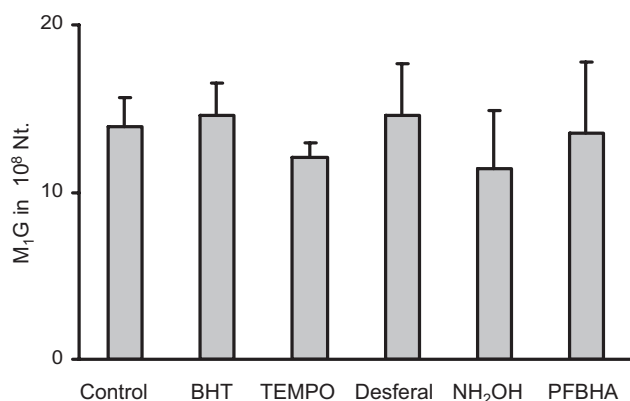
In order to investigate whether there was artifactual formation of M<sub>1</sub>G adducts from oxidation of deoxyribose or lipids that could be prevented in part by antioxidants, DNA was prepared from rat liver tissue with or without the addition of antioxidants such as BHT or TEMPO. As shown in Figure 1, supplementing DNA isolation reagents with BHT or TEMPO did not make a statistically significant difference regarding the number of M<sub>1</sub>G adducts in DNA compared with the control DNA isolated without an antioxidant. Since desferal, a metal ion chelator, has been shown to significantly reduce the artifactual formation of 8-OH-dG (30), the effects of its presence during DNA isolation on the number of M<sub>1</sub>G adducts was tested. In addition, hydroxylamine or PFBHA was tested because they have high reactivity toward MDA and base propenals (i.e. precursors of M<sub>1</sub>G). Neither desferal nor alkoxyamines resulted in a statistically significant change in the number of M<sub>1</sub>G adducts in the DNA compared with that of control. The effect



**Figure 1.** The effect of antioxidants or temperature on the number of M<sub>1</sub>G adducts after DNA isolation from control tissue. DNA was isolated from control rat liver tissue with the addition of antioxidant at room temperature (Method A, open rectangles) or at 4°C (Method B, closed rectangles). The number of M<sub>1</sub>G adducts in the DNA was quantified with PFBHA labeling for M<sub>1</sub>G followed by LC-MS/MS analysis of PFBHA-M<sub>1</sub>G conjugates as detailed in the Materials and Methods. Each point represents mean  $\pm$  SD from five to six samples for each group. N.T.: not tested.

of a low temperature work-up for DNA isolation has been tested with combinations of different types of antioxidants, a chelator or alkoxyamines. It has been well established that work-up procedures at low temperature significantly reduce the oxidation of unmodified DNA molecules to produce primary oxidative lesions (i.e. 8-OH-dG, single strand breaks and AP sites) during DNA isolation (20,31,32). In contrast to the results for primary oxidative lesions, neither low temperature nor the addition of antioxidant made a significant difference compared with control (Figure 1).

In parallel experiments, the loss of M<sub>1</sub>G during DNA isolation was tested using positive control tissue nuclear fractions. Hydroxylamine or PFBHA was reacted with M<sub>1</sub>G in DNA to prevent binding with proteins or amines that could result in the reduced recovery of M<sub>1</sub>G after DNA isolation. As shown in Figure 2, all additives including hydroxylamine and PFBHA



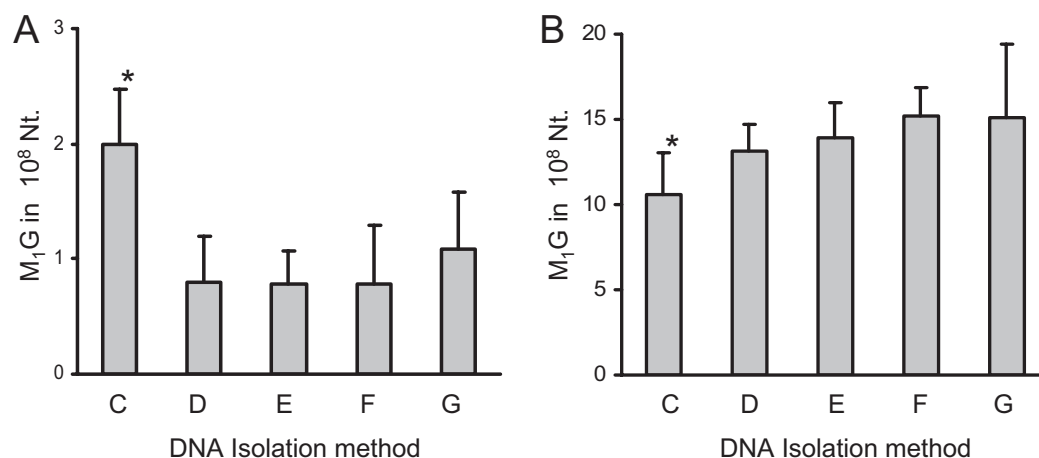
**Figure 2.** The effect of antioxidants on the number of M<sub>1</sub>G adducts after DNA isolation from positive control tissue. DNA was isolated from positive control samples consisting of MDA treated CTD and the nuclear fraction prepared from rat liver tissue. The antioxidants or alkoxyamines were added into the reagents for DNA isolation (Method A). Each point represents mean ± S.D. from five samples for each group.

had no significant effect on the number of M<sub>1</sub>G adducts in the DNA compared with control. Based on the number of M<sub>1</sub>G adducts detected in samples, the recovery of positive DNA was 59 ± 12% from all samples. No statistical significance was observed compared with controls which did not have any additives during DNA isolation.

### Comparison of DNA isolation methods for M<sub>1</sub>G adducts analysis

Artificial formation or loss of M<sub>1</sub>G during DNA isolation was further investigated by a comparison of different DNA isolation protocols. TEMPO was used as an antioxidant because of its solubility in both water and organic solvents. As demonstrated in Figure 3A, DNA isolation from tissue homogenates that did not involve nuclear fraction collection (Method C) resulted in a statistically significant increase in the number of M<sub>1</sub>G adducts when compared with DNA isolated from nuclear fraction (Methods D, E, F and G). There was no statistical difference between the other four groups (Methods D, E, F and G) as indicated by the ANOVA. Student *t*-test resulted in a significant difference ( $P < 0.05$ , two tails) in the number of M<sub>1</sub>G between Method C and all other groups. DNA isolation with Tris as a buffering agent did not result in a statistically significant difference in the number of M<sub>1</sub>G adducts compared with the result obtained from DNA isolated with HEPES. Two different DNA isolation protocols using NaCl or NaI to remove proteins did not show any statistically significant difference in the number of M<sub>1</sub>G adducts when compared with DNA isolation with phenol extraction. In the experiment to isolate DNA from positive control samples, Method C resulted in a significant reduction in the number of M<sub>1</sub>G adducts in the DNA compared with Methods E and F. There was no statistically significant difference among the other four groups (Figure 3B).

In addition to M<sub>1</sub>G numbers, DNA yields and purities were determined and compared among the different DNA isolation



**Figure 3.** Comparison of DNA isolation methods on the number of M<sub>1</sub>G adducts after DNA isolation. DNA was isolated from control (A) or positive control (B) using different types of buffering reagents or methods for the removal of protein. The details regarding DNA isolation are described in the Materials and Methods. Method C: DNA isolation without a preisolation of nuclear fraction, Method D: protein extraction with Tris-buffered phenol, Method E: protein extraction with HEPES-buffered phenol, Method F: protein removal with salting-out, Method G: protein removal with NaI. Each point represents mean ± SD from five samples for each group. Asterisk, statistically significant difference from other groups ( $P < 0.05$ ).

**Table 2.** Comparison of DNA yield and purity isolated from control rat liver with different methods

DNA isolation method	A <sub>260</sub> /A <sub>280</sub>	DNA yield based on A <sub>260</sub> (mg/g tissue)	Nucleotides (μmol nt/mg DNA)	Calibrated DNA yield (μmol/g tissue)
C	1.79 ± 0.04	0.95 ± 0.04	2.51 ± 0.46	2.40 ± 0.46
D	1.79 ± 0.02	1.00 ± 0.05	2.69 ± 0.10	2.70 ± 0.11
E	1.79 ± 0.03	0.99 ± 0.05	2.45 ± 0.27	2.42 ± 0.29
F	1.80 ± 0.03	0.93 ± 0.01	2.86 ± 0.06 <sup>a</sup>	2.65 ± 0.05
G	1.71 ± 0.02 <sup>a</sup>	0.77 ± 0.09 <sup>a</sup>	2.58 ± 0.40	1.96 ± 0.17 <sup>a</sup>

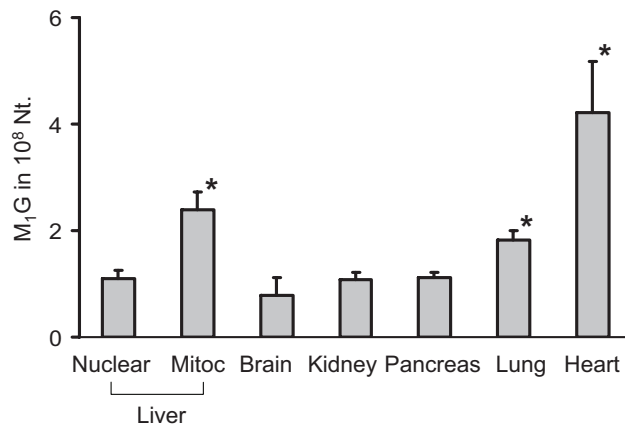
Each point represents mean ± S.D. from five samples for each group.

<sup>a</sup>Statistically significant difference from other groups ( $P < 0.05$ ).

methods. From a visual inspection of DNA pellets and solutions, DNA isolated with Method G was different in color possibly owing to a presence of iodine in the DNA. As detailed in Table 2, Method G resulted in a statistically significant difference in the A<sub>260</sub>/A<sub>280</sub> ratio compared with that of other DNA isolation methods. The purity tested with UV absorbance (A<sub>260</sub>/A<sub>280</sub> ratio) for the DNA isolated with Methods C, D, E and F was comparable with 1.8. The phenol extraction methods (Methods C, D and E) produced the best yield. Method F resulted in DNA yields comparable with the phenol extraction methods. However, Method G resulted in significantly decreased yield for DNA recovery compared with other methods. The purity of the DNA was further tested by HPLC analysis for purine bases, as part of the M<sub>1</sub>G adducts assay. The number of total bases in each sample was calculated based on A-T and G-C base pairing. The results are described in Table 2. The theoretical value for the number of nucleotides from 100 μg DNA was 302 nmol based on the average molecular weight of dNMP in double-stranded DNA (331: dNMP – H<sub>2</sub>O + Na<sup>+</sup>) and assuming a 1:1 ratio of Gua to Ade. The highest yield for purine bases was 2.85 μmol nt/mg DNA, which corresponds to 95% recovery from DNA isolated with Method F. The DNA prepared with Methods C, D, E and G showed significantly lower recovery of purines, which suggests more impurities in those DNA samples. The DNA yield was recalculated from the results of purine analyses. Methods D and F produced the best yield in DNA isolation followed by Methods E and C. The DNA yield from Method G was significantly less than that of the other methods. Based on the result of DNA purity, yield, and the number of M<sub>1</sub>G adducts in the DNA, Method F was selected as a standard DNA isolation protocol for the M<sub>1</sub>G adducts assay because of consistent results without significant artifact.

### DNA isolation from rat tissues

Using Method F, nuclear DNA or mitochondrial DNA was isolated from different tissues of male rats. As shown in Figure 4, mitochondrial DNA from liver tissue showed about a 2-fold greater number of M<sub>1</sub>G adducts compared with liver nuclear DNA (1.1 M<sub>1</sub>G/10<sup>8</sup> nt). The nuclear DNA isolated from brain, kidney and pancreas contained a comparable number of M<sub>1</sub>G adducts to liver nuclear DNA. However, the nuclear DNA isolated from the lung or heart contained a 1.7- or 3.8-fold greater numbers of M<sub>1</sub>G adducts, respectively, compared with liver nuclear DNA.



**Figure 4.** The number of M<sub>1</sub>G adducts in liver mitochondrial DNA or nuclear DNA isolated from different rat tissues. Each point represents mean ± SD from four samples for each group. Asterisk, statistically significant difference from liver nuclear DNA ( $P < 0.05$ ).

### DISCUSSION

The goal of the present study was to establish a reliable protocol for DNA isolation from animal tissues with the following criteria: (i) to minimize artifactual formation of M<sub>1</sub>G from unmodified DNA bases and (ii) to maximize the recovery of M<sub>1</sub>G during DNA isolation procedures.

The initial approach to investigating the artifactual formation of M<sub>1</sub>G involved determining the effect of antioxidants, a chelating reagent, aldehyde reactive alkoxyamines or different DNA isolation methods on the number of M<sub>1</sub>G adducts using rat liver tissue. Transition metals or oxidized phenols have been shown to generate oxygen radicals and probably represent the main mechanism for artifactual increases in oxidative DNA lesions. A number of researchers (20,31,32) examined the protective effects of antioxidants to test the hypothesis that oxidative DNA lesions including 8-OH-dG, aldehydic DNA lesions (ADLs) and strand breaks are artifactually produced during DNA isolation. In addition, low temperature work-up procedures or DNA isolation with non-phenol extraction methods (33) showed significantly lower numbers of 8-OH-dG. However, the results from the present study clearly demonstrate that DNA isolated from the nuclear fraction has consistent numbers of M<sub>1</sub>G adducts regardless of which method of isolation is used, and whether or not antioxidants are added. It was hypothesized that the overall process of producing M<sub>1</sub>G, compared with primary oxidative DNA lesions, requires more time and ROS in order to produce MDA or precursors to react with Gua. Therefore, the formation of secondary ROS damage is less probable to occur during DNA isolation or sample processing compared to that of primary ROS lesions.

The present study also investigated the loss of M<sub>1</sub>G during DNA isolation by introducing protective reagents, such as hydroxylamine and PFBHA, and by comparing with positive control DNA. No statistically significant difference was observed between DNA isolation with or without PFBHA, which suggests that the loss of M<sub>1</sub>G during DNA isolation is not a critical issue, at least in the protocols that were used.

To further verify these results, the present study compared M<sub>1</sub>G numbers in CTD either purchased from Sigma (St Louis,

MO) or isolated freshly from frozen tissue. The purchased CTD contained orders of magnitude greater numbers of primary oxidative DNA lesions such as 8-OH-dG, AP sites and single strand breaks compared with the carefully isolated CTD (S. Yamaguchi, P. H. Lin, J. Nakamura, S. Liao and J. A. Swenberg, unpublished data). However, the two different CTD had comparable numbers of M<sub>1</sub>G adducts.

In an effort to establish the normal range of M<sub>1</sub>G adducts *in vivo*, the present study compared different organs regarding the number of M<sub>1</sub>G adducts. Surprisingly, the number of M<sub>1</sub>G adducts in liver nuclear DNA was 40- to 3000-fold lower than it had been indicated in previous work (12,15–17). The varying numbers of primary oxidative DNA lesions reported by different researchers stem from artifacts during DNA preparation and sample analysis as indicated in the review by Collins *et al.* (34). However, the artifacts during DNA isolation were extensively investigated in this study and turned out to be much less problematic for M<sub>1</sub>G adducts analysis. Therefore, the discrepancy between our results and other references with regard to the number of M<sub>1</sub>G adducts is most probably owing to different methods of measurement. As detailed previously (18), the novel M<sub>1</sub>G adduct assay involving the selective labeling of aldehydic adducts with subsequent LC–MS/MS analysis affords a sensitive and specific means of M<sub>1</sub>G adduct measurement. However, some of the values reported in previous references were obtained either from HPLC-fluorescence measurements (15), <sup>32</sup>P-postlabeling (12) or immunoslot assays (17) that may not have been specific enough to measure extremely low amounts of this DNA adduct. Although GC–MS measurement can provide very specific detection of DNA adducts, the artifacts that arise during DNA hydrolysis or derivatization cause a significant increase in the number of oxidative lesions such as 8-OH-dG (31,35). In addition, GC–MS analysis of M<sub>1</sub>G adducts (16) after derivatization had interference at low femtomole amounts owing to the noise from the instrument, as mentioned elsewhere (13), which showed an overestimation for the number of M<sub>1</sub>G adducts in blank samples as 40 fmol. Therefore, the present study suggests that the numbers of M<sub>1</sub>G adducts in genomic DNA have been overestimated owing to the insufficient specificity of previous measurement methods.

Compared with other oxidative DNA lesions such as 8-OH-dG (36), or abasic sites (24), the numbers of M<sub>1</sub>G in genomic DNA isolated from rat tissues were one to two orders of magnitude lower in all tissues. These results are consistent with the results from *in vitro* experiments (37) showing that the formation of M<sub>1</sub>G was an order of magnitude lower than that for 8-OH-dG or ADLs in ROS reactions with DNA. However, the change in the number of M<sub>1</sub>G in the DNA exposed to oxidative conditions was similar to the change in the number of primary ROS lesions (37).

The results regarding the numbers of M<sub>1</sub>G adducts in DNA isolated from diverse types of tissues showed a clear difference in the amount of oxidative DNA damage from tissues or cell compartments. The difference in the number of M<sub>1</sub>G adducts in the nuclear DNA from six tissues showed that M<sub>1</sub>G is related to the amount of ROS production. Rolfe *et al.* (38) reported that the heart uses more oxygen and produces ATP at a faster rate than the liver does. The results from this study were closer to the results regarding ADLs (24) rather than 8-OH-dG reported by other groups (36). This result is in good

agreement with the hypothesis that oxidative DNA sugar damage plays an important role in M<sub>1</sub>G formation (37). Consistent with this hypothesis, the number of M<sub>1</sub>G adducts was significantly greater in mitochondrial DNA compared with nuclear DNA. It has long been recognized that mitochondrial DNA is more prone to oxidative DNA damage owing to the high amount of ROS (36,39–41). In addition to the high amount of ROS in the mitochondria, the deficiency of nucleotide excision repair in mitochondria could be responsible for the difference in M<sub>1</sub>G numbers between mitochondrial DNA and nuclear DNA (42,43) since nucleotide excision repair has been reported to be an important pathway for M<sub>1</sub>G repair for nuclear DNA (44).

In conclusion, the results from the present study suggest that M<sub>1</sub>G is less prone to oxidation caused by DNA isolation. However, it is still sensitive to ROS damage within living cells. Therefore, M<sub>1</sub>G should be a good biomarker for monitoring oxidative DNA damage, which may play a role in numerous genetic diseases including aging and cancer.

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